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X-Inactivation Patterns in Monozygotic and Dizygotic Female Twins

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We have tested the hypothesis that contrasting X-inactivation patterns could be a trigger for monozygotic twinning in females. X-inactivation patterns were studied in umbilical cord tissue in 43 monozygotic twin pairs and 24 dizygotic twin pairs. Very skewed or non-random X-inactivation patterns were observed in both twins in six of the monozygotic twin pairs and in one of the dizygotic twin pairs. Contrasting X-inactivation patterns occurred in only one of the six monozygotic twin pairs. This does not support the original hypothesis. There is a trend to extreme skewing of X-inactivation pattern occurring more frequently in monozygotic twins. © 1996 Wiley-Liss, Inc.

KEY WORDS: X-inactivation, twins, monozygotic twins

INTRODUCTION

Monozygotic twins can be dichorionic diamniotic, monochorionic diamniotic, monochorionic monoamniotic, or conjoined depending on the timing of separation. There is a female excess in monozygotic twins, particularly in later dividing twins and most obvious in conjoined twins where the female to male ratio is 2:1 [James, 1980]. This has led to the hypothesis that aberrant X-inactivation patterns could be a trigger for the twinning event [Burn et al., 1986]. There have been a number of recent reports of female twins who are discordant for X-linked disorders with one twin fully manifesting the disorder [Harris, 1983; Mayo et al., 1971; Pena et al., 1987; Bonilla et al., 1990; Jongbloet, 1983; Hay and Wehrung, 1970; Burn et al., 1986; Winchester et al., 1992; Levade et al., 1991]. The explanation for most of these cases is that the affected twin has a non-random X-inactivation pattern. However, it is not known whether the incidence of females manifesting X-linked disorders is higher in monozygotic twin pairs than in singletons or whether there has been reporting bias. In an attempt to clarify this we circulated a questionnaire to delegates at the European Workshop on Germline Mosaicism in Duchenne Muscular Dystrophy [Van Essen et al., 1992] asking for numbers of monozygotic twins who are carriers of Duchenne muscular dystrophy on their registers and for clinical information about them.

We have carried out a prospective study of X-inactivation patterns in a cohort of normal female twins to compare the X-inactivation patterns in monozygotic twins with the dizygotic twins acting as the control group. The X-inactivation patterns were investigated using the methylation differences between the active and inactive X chromosomes that occur at a HpaII site, twenty base pairs from a polymorphic (CAG)n trimeric repeat in the androgen receptor locus [Allen et al., 1992]. Zygosity was established by standard methods.

METHODS

The questionnaire to European Departments of Genetics yielded five female monozygotic twin pairs who were carriers of Duchenne muscular dystrophy that had not been reported previously. Two twin pairs had no clinical symptoms, one twin in two of the twin pairs had a positive Gower's manoeuvre by the age of 10, and one twin in the final twin pair now has problems going up stairs at the age of 40. We have studied X-inactivation patterns in lymphocyte DNA from two of the discordant twin pairs and the third has been investigated by Simone Gilgenkrantz. One of the twin pairs was heterozygous for the simple sequence repeat at the androgen receptor locus and investigated by this method. The second twin pair was homozygous at this locus but heterozygous for a polymorphism at the PGK locus. X-inactivation analysis at this locus was carried out as described previously [Vogelstein et al., 1987].

Placentae and umbilical cords were collected from 106 female twin deliveries. Cord tissue was homogenised, after expelling cord blood, and DNA extracted by the guanidine precipitation method. The cord blood samples were used for typing red cell antigens (ABO, rhesus, MNSs, Colton, Kell, Duffy, Kidd, and Xg) and biochemical markers (adenosine deaminase, esterase D, phosphoglucomutase, acid phosphatase, glyoxylase,

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glutamic-pyruvic transaminase, properdin factor B, and group-specific component) by standard methods [Race and Sanger, 1975]. In cases where no cord blood was obtained, cord DNA was analysed using 6 microsatellite polymorphisms. Probability of monozygosity was calculated using Bayes theorem as reviewed by Burn and Corney [1988].

X-inactivation patterns were studied as follows: 1.5 μg DNA from each cord was digested with 10 units HpaII in 10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM dithiothreitol in a total volume of 30 μl. Following overnight incubation at 37°C the digest was heated to 100°C for 5 minutes, centrifuged for 5 seconds, and 8 μl of supernatant used for PCR as described below. PCR was performed on a 400 ng aliquot of DNA from each cord in a final volume of 50 μl containing 35 pmol of each oligonucleotide primer (5′-TTCCAGAATCTGTTCCAGAGCG-3′ and 5′-TGAAGGTTGCTGTTCCTCATCC-3′), 200 μM each dNTP, 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X, and 2 units TaqXL (Northumbria Biologicals Ltd.). The first amplification cycle was 94°C for 3 minutes, 52°C for 2 minutes and 72°C for 1 minute. Thirty-four cycles of 94°C 1 minute, 52°C 1 minute, 72°C 1 minute were performed increasing the extension period by 3 seconds/cycle and adding a final extension step of 10 minutes. Alleles were visualised in a non-denaturing 6% polyacrylamide gel stained with ethidium bromide. Photographs of the gels were scored independently by two investigators as random, skewed, very skewed, or non-random pattern (Fig. 1). The HpaII digest and PCR were repeated on all samples that gave a very skewed or non-random pattern. Zygosity information was not given to the investigators scoring the X-inactivation studies.

RESULTS

One of the twin pairs discordant for clinical signs of Duchenne muscular dystrophy was informative for the androgen receptor polymorphism. The twin with early onset of symptoms had a non-random X-inactivation pattern and her asymptomatic twin had skewing of the X-inactivation pattern in the same direction. The twin pair in which one twin has weakness in her forties was heterozygous for the polymorphism at the PGK locus. Both the symptomatic and asymptomatic twin had random X-inactivation patterns in DNA extracted from blood.

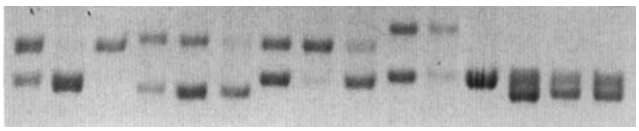

Fig. 1. This figure shows the results from 5 twin pairs. Each twin pair is represented by 3 tracks. The first track shows cord DNA amplified directly and demonstrates the polymorphism. The second and third tracks show DNA from each twin amplified after HpaII digestion. The first twin pair shows contrasting use of the X chromosome, both with a very skewed pattern. One twin of the second pair has a random pattern and the other a very skewed pattern. The twins of the third pair show contrasting skewing. One twin of the fourth pair has a random pattern and the other twin a non-random pattern. Both twins in the fifth pair have a random X-inactivation pattern.

Sixty-seven of the 106 twin pairs were heterozygous for the (GAC)n polymorphism at the androgen receptor locus. Of these, 43 were monozygotic (MZ) twins and 24 were dizygotic (DZ). One twin was heterozygous in a further 6 dizygotic twin pairs. The results of the X-inactivation studies are given in Tables I and II. Contrasting non-random X-inactivation patterns were seen in one MZ twin pair and no DZ twin pairs. Extreme skewing in the same direction was observed in 6 twin pairs, five MZ and one DZ. Considering the twins as individuals, 16 of 86 MZ twins (18.6%) displayed extreme skewing compared with 5 of 54 DZ twin individuals (9.3%).

ANALYSIS

The results did not support the original hypothesis that contrasting non-random use of the X is a cause of MZ twinning. There was considerable variation from a random pattern of X-inactivation, with 43% of all MZ twin individuals and 31% of all DZ twin individuals showing some degree of skewing. Of greater clinical and biological relevance is the proportion showing extreme skewing or non-random pattern. The proportion 16/86 (18.6%) in MZ twins is not significantly greater than the 5/54 (9.3%) among DZ twins using simple chi-squared analysis. However, this is a somewhat misleading analysis since the number of DZ twins available for study was limited by the design of the study. If the data from the MZ twins are treated as a binomial and the observed proportion of 16/86 is compared to an estimate that 6% of all normal females show extreme skewing of X-inactivation, the excess is highly significant ($P < 0.001$). Even taking a figure of 10%, the proportion among MZ twins is significantly elevated. The 95% confidence limits for extreme skewing in MZ twin individuals based on the present study are 10.38–26.82%, assuming the variance to be normally distributed.

TABLE I. X-Inactivation Patterns in MZ and DZ Twin Pairs*

X-inactivation pattern	Monozygotic	Dizygotic
Both random	18	13
1 skewed	9	7
Both skewed ↑↑	4	—
Both skewed ↑↓	2	—
1 random, 1 very skewed	1	—
1 random, 1 very skewed ↑↓	—	2
Both very skewed ↑↑	3	—
Both very skewed ↑↓	—	—
1 random, 1 non-random	1	—
1 very skewed, 1 non-random ↑↑	2	1
1 non-random, 1 non-random ↑↓	1	—
Total	43	24

*Twins in the MZ column had a greater than 99% probability of monozygosity as calculated by Bayes theorem. The DZ column includes information from the DZ twin pairs, where both twins were heterozygous for the polymorphism at the androgen receptor locus used to study X-inactivation patterns. The arrows indicate the direction of the skewing. If the arrows point in the same direction it indicates the direction of skew was the same in both twins, whereas arrows pointing in opposite directions indicate that one twin has preferential use of the maternal X and the other has preferential use of the paternal X.

TABLE II. X-Inactivation Patterns in MZ and DZ Twin Individuals*

X-inactivation pattern	MZ	DZ
Random	49	37
Skewed	21	12
Very skewed	11	4
Non-random	5	1

*This table summarises the X-inactivation patterns seen in individuals from the monozygotic and dizygotic twin pairs. Information is included from the dizygotic twins, where only one of the pair was heterozygous for the polymorphism used to study the X-inactivation patterns. MZ, monozygotic; DZ, dizygotic.

DISCUSSION

Contrasting non-random X-inactivation patterns occurred in only one twin pair. These twins were monozygotic. In the 6 remaining twin pairs where both had extreme skewing of the X-inactivation pattern (5 monozygotic and 1 dizygotic) the direction of skewing was the same in both twins. These observations do not support the original hypothesis that aberrant X-inactivation leading to clumps of cells with contrasting X-inactivation patterns is a frequent cause of monozygotic twinning in females.

The results of the questionnaire survey of the muscular dystrophy registers in Europe documented 5 previously unreported MZ Duchenne twin carriers. Three of these had one manifesting carrier, two of whom had childhood onset. Our investigation of one twin with early onset of symptoms showed a non-random X-inactivation pattern; her asymptomatic twin had a pattern skewed in the same direction. In the twin pair in which one twin has weakness in her 40s both twins have random X-inactivation patterns in DNA extracted from blood. X-inactivation studies in the French pair studied by Gilgenkrantz documented contrasting use of the X chromosome in the two twins [Tremblay et al., 1993]. The observation of 2/10 girls with clinical signs in childhood in these twin pairs suggests that the cases in the literature do not simply reflect reporting bias. The figures from the survey are in keeping with our finding that 19% of monozygotic twin females have very skewed or non-random use of the X. The incidence of manifesting carriers may be higher in MZ twins than the 2.5% reported in female carriers of Duchenne muscular dystrophy [Norman and Harper, 1989], though the numbers are too small to reach statistical significance.

When Table I was assembled it was obvious that there was no support for the prediction that there would be a significant number of MZ twins with contrasting X-inactivation pattens, but it was clear that skewing of X-inactivation is common and that there was a trend towards a greater degree of skewing in MZ twins. The analysis based on Table II where twins are considered as individuals shows that while formal Chi-squared analysis of the MZ versus the DZ population was non-significant, the proportion of MZ individuals showing extreme X-inactivation is significantly higher than literature estimates of its frequency in the general female population. Unfortunately there is no published report of a large series of normal females studied by this method. In a recent study of cultured fibroblasts from 48 female patients at risk of pyruvate dehydrogenase E1 alpha subunit deficiency and 20 normal controls 25% of both patients and controls had a greater than 80:20 skew [Brown and Brown, 1993]. Puck investigated 37 normal females by making multiple T-cell/hamster fibroblast hybrid cell clones and then studying X-inactivation using methylation sensitive RFLPs [Puck et al., 1992]. She found a range of paternal X chromosome use between 20 and 86% in these women, with 35 of them falling between 25 and 75%. Another approach to estimates of extreme skewing in females is to look for manifestation of X-linked diseases. The largest study of this type is that carried out by Thuline and Buckley [Thuline, 1964] who found 6.2% of males and 0.55% of females to be red-green colour blind in a series of over 10,000 students. Allowing for homozygous females, this would suggest that 1 to 2% of females manifest because of extreme skewing of X-inactivation, in keeping with the findings of Norman and Harper that 33/119 (2.5%) of female carrier of Duchenne muscular dystrophy in Wales manifest the disease. Taking the literature into account we conclude that there is a high probability that MZ twins are unusually prone to this phenomenon and that this is reflected by the number of reports of MZ twins manifesting X-linked diseases.

If the greater frequency of extreme skewing among MZ females is confirmed, it would suggest that X-inactivation in MZ twins is occurring when the number of cells in each progenitor pool mass is smaller. The elegant studies of [Tan et al., 1993] have provided supporting evidence that X-inactivation occurs at the time of differentiation of each tissue. If this is the case, the present study would suggest that MZ twins have a tendency to initiate differentiation at the same time post-fertilization as a singleton but with a reduced cell mass. Reduced cell volume has been invoked as one explanation for the excess of malformations in MZ twins. Whether or not this is the case, it is clear that the study of MZ twins continues to provide an interesting insight into early human development.

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